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Gene in Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) The ANX7 gene codes for a Ca ²⁺ -activated GTPase, and is a tumor suppressor gene. Loss of ANX7 expression is associated with hormone refractory metastatic prostate cancer patients. Our <u>objective</u> in this proposal is to determine the mechanism and the signaling pathway by which the ANX7 over-expression can induces death of prostate cancer cells. To this end, we have generated and identified the dominant negative mutants against calcium and GTP in the anx7 coding domain which are necessary for biochemical functions of ANX7. We also have generated the effective adenoviral constructs containing the dominant negative mutants and wt-ANX7 and have shown that altered ANX7 expression during prostate cancer cell growth involves calcium. Additionally, we confirmed the role of calcium by determining ANX7's control on all three subtypes of IP3 Receptor expression. Using cDNA microarray analysis, we have identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of prostate cancer cell growth. Taken together, these data indicate that ANX7 suppresses prostate cancer cell growth and calcium plays a role via IP3-Receptor. In addition, we have identified the members of the ANX7 signaling pathway. Comprehensive effort is underway to validate the results that will further elucidate the ANX7 dependent calcium signaling pathway in prostate cancer cells.				
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INTRODUCTION

Current attempts to develop more effective therapies for prostate cancer have been focussed on the discovery of new tumor suppressor genes, in hopes of using them for gene therapy. We have recently found that the *ANX7* gene, which we discovered and have continued to study in the context of Ca^{2+} and GTP mediated exocytosis for many years (Creutz et al, 1978; *ibid*, 1979; Raynal and Pollard, 1994; Caohuy et al, 1996; Srivastava et al, 1996), is defective in 35% of human prostate cancer specimens (Srivastava et al., 2001a) and 40% of human breast cancer specimens. A detailed analysis of *ANX7* levels in hundreds of prostate cancer specimens reveals that expression of this candidate tumor suppressor gene is specifically altered in metastatic and hormone refractory prostate cancers (Srivastava et al., 2001a). These data therefore, strongly suggest that the *ANX7* gene has clinical relevance for prostate cancer in men. This is an important insight because until now the *ANX7* gene has never been thought to play such a role (Raynal and Pollard, 1994). In our preliminary studies with metastatic prostate cancer cells in vitro, we find that *ANX7* gene therapy causes these cells to undergo apoptosis, or programmed cell death. Therefore our objective in this proposal is to determine the mechanism and the signaling pathway by which the *ANX7* gene induces death of prostate cancer cells. The rationale behind this study is the hope that such knowledge will enable us to develop therapies based on the use of the *ANX7* gene to treat or prevent prostate cancer. We are best positioned to pursue this work because we not only discovered the *ANX7* gene, but have also recently prepared a tumor-prone *Anx7* knockout mouse. It has fascinated us that the phenotype of this knockout mouse also includes such a high frequency (20-25%) of spontaneous tumors.

Our studies with both human cancer cells and the *Anx7* knockout mouse have indicated the existence of a possible common deficit in calcium regulation (Srivastava et al., 1999). In the case of cancer cells, it is known that the initial signal for the onset of programmed cell death is the release of a pulse of calcium from the internal calcium stores in the endoplasmic reticulum. The calcium is released into the cytoplasm through a protein channel in the endoplasmic reticulum membrane called the IP3-Receptor. A hint regarding the possible connection between the *ANX7* gene and the calcium pulse, comes from our recent work on the *ANX7* (+/-) knockout mouse. Tissues in this mouse express low levels of *Anx7* protein as well as low levels of IP3-Receptors in the endoplasmic reticulum. Intracellular calcium signaling is accordingly defective, in spite of normal levels of extracellular calcium. We are reminded that apoptosis, from whatever cause, is known to be suppressed in the absence of IP3-Receptors. We also found that the effect of the *ANX7* gene on the DU145 prostate cancer cell line enhanced the capacity for undergoing apoptosis in the presence of staurosporine. Additionally, transfection of wild type *ANX7* in adenovirus system enhanced the production of IP3-Receptors, while dominant negative mutant of *ANX7* suppressed IP3-Receptor expression. On the basis of these results **we have hypothesized that the *ANX7* gene kills prostate cancer cells by increasing IP3-Receptor expression, thereby potentiating the IP3-dependent apoptotic calcium signaling pathway.**

BODY

Reason for the change in the statement of work for Aim #1 and 2. To conduct experiments efficiently in a timely manner, first we concentrated in accomplishing the SOW 2.a. That is to test sites of Ca^{2+} , GTP and PKC on ANX7 for importance in induction of apoptosis. Since we can test both the wild type and best characterized mutants against calcium, GTP and PKC for apoptosis and tumor suppression simultaneously and accomplish SOW 1.a, I request a change in the time line for Aim #1 and 2

STATEMENT OF THE THE WORK:

TIME-LINE FOR EXPERIMENTAL PLAN (OLD PLAN)

	<u>YEAR #1</u>	<u>YEAR #2</u>	<u>YEAR#3</u>
<u>Aim#1 and 2.</u>			
1.a. Test tumor cells for biochemical characteristics of apoptosis	-----		
2.a. Test sites of Ca^{2+} , GTP and PKC on ANX7 for importance in induction of apoptosis		-----	
2.b. Test tumorigenicity of MCF7 cells expressing wildtype and mutant ANX7 in cancer cells			-----

TIME-LINE FOR EXPERIMENTAL PLAN (NEW PLAN)

	<u>YEAR #1</u>	<u>YEAR #2</u>	<u>YEAR#3</u>
<u>Aim#1 and 2.</u>			
1.a. <u>Test sites of Ca^{2+}, GTP and PKC on ANX7 for importance in induction of apoptosis</u>	-----		
2.a. <u>Test tumor cells for biochemical characteristics of apoptosis</u>		-----	
2.b. Test tumorigenicity of MCF7 cells expressing wildtype and mutant ANX7 in cancer cells			-----

Milestone #1: Elucidation of the relationship between ANX7-induced alteration of apoptosis and tumor suppressor activity in prostate cancer cells

Milestone #2: Defining the biochemical requirements and regions in ANX7 that confer apoptosis and tumor suppressor activity in cancer cells

Aim #3.	<u>YEAR #1</u>	<u>YEAR #2</u>	<u>YEAR#3</u>
3.a. Test whether ANX7 increases IP3- Receptor expression in tumor cells	_____		
3.b. Determine subcellular localization of induced IP3-Receptors		_____	
3.c. Study the physiological consequences of ANX7 expression on mobilization of intracellular Ca ²⁺ in DU145 cells expressing wildtype and mutant ANX7			_____

Milestone #3: We will investigate the mechanism by which ANX7 permits a pro-apoptotic calcium pulse to be emitted into the prostate cancer cell cytosol

Aim #4.

4.a. Perform cDNA microarray studies on cells
expressing wildtype and mutant *ANX7* _____

Milestone #4: We will identify the signaling pathway by which ANX7 suppresses prostate cancer cell growth and the downstream targets that are involved in causing apoptosis

In the first year of the grant proposal we have focused on the generation of adenovirus constructs containing wild type ANX7 and the dominant negative mutant against calcium and GTP. We investigated the anticarcinogenic effects of the altered ANX7 expression (**SOW for Aim #1 and 2**) and studied the expression of all three IP3-Receptors (**SOW for Aim #3**). We also began studies on the signaling pathway using cDNA microarray to gain possible mechanism for ANX7's inhibition of growth (**SOW for Aim 4**)..

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To define the biochemical requirements of ANX7 for apoptosis and tumor suppressor activity in prostate cancer cells, we will determine the "hot spot(s)" in the *ANX7* coding domain which are necessary for tumor cell proliferation and apoptosis.

The transformants with the 'tet off' system (from Clonetech) bearing wildtype ANX7 gene had problem of leakage in the absence of tetracycline. So we switched to adenoviral system. *ANX7* is expressed at very low levels in prostate cancer cells. Hence it is critical to generate adenoviral expression vectors which has high transfection efficiency to study the biological functions of *ANX7*. To determine the involvement of calcium, GTP and PKC, we first generated mutations at those sites in mammalian pTrc99 vector and tested in vitro the ANX7 activity for membrane fusion. Therefore, we generated expression vectors containing wt-ANX7, 16 mutants against calcium binding site, 8 mutants against GTP binding site and 3 mutants against PKC binding site to study

the biological functions of ANX7. The selected dominant negative mutants were reconstructed into adenovirus system and used for its activity for tumor suppression, apoptosis and IP3-Receptor expression and other downstream targets and were compared with wt-ANX7.

1.a.1. Generation of dominant negative mutants of ANX7.

Rationale: Dominant negative mutants of tumor suppressor genes have been useful for investigating the mechanism of action of these genes. Since it is well known that ANX7 forms Ca^{2+} -dependent polymers as a prelude to membrane interactions, it seemed reasonable to anticipate that mutations affecting Ca^{2+} binding might have the ability to generate partially crippled ANX7 monomers in the polymerization reaction. We reasoned that if a mutant could be found that inhibited polymerization, we could test whether tumor suppressor activity depended on this process.

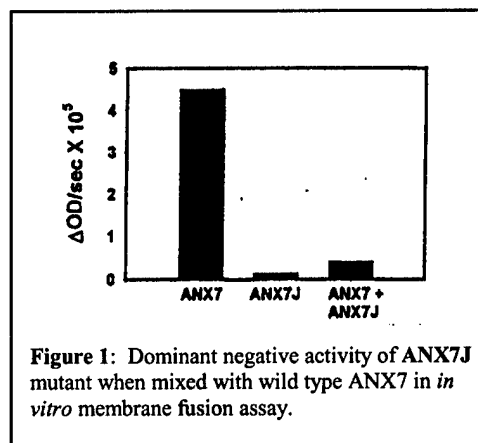


Figure 1: Dominant negative activity of ANX7J mutant when mixed with wild type ANX7 in *in vitro* membrane fusion assay.

Hypothesis: Mutations at some or all of the four Ca^{2+} binding sites on ANX7 may act as dominant negative mutants.

Experiment: Using standard techniques, site directed mutations were introduced into the calcium binding sites in combinations of all four crystallographically defined endonexin fold motifs. All four have the consensus sequence [GXGTDE] and the mutations were engineered to generate the amidated analogues of the charged residues (*viz.*, [GXGTNQ]). Thus we prepared 16 different combinations, including the wildtype ANX7. The combinations were single mutations (e.g., 1, 2, 3 or 4); mutations at two sites (e.g., 1 & 2, 1 & 3, etc); mutations at three sites (e.g., 1&2&3, 2&3&4, etc) and all four sites (e.g., 1&2&3&4). All the mutants were prepared and tested in the phosphatidylserine liposome fusion assay. Some were as active as the wildtype, while others were much less active. As shown in **Figure 1**, one mutant, ANX7J, was both intrinsically inactive, and profoundly inhibitory when mixed with equi-molar amounts of wildtype ANX7 (*viz.*, 1 μg each of ANX7 proteins).

Interpretation: ANX7J behaves as a dominant negative mutant in the *in vitro* test, and is now ready for testing for tumor suppressor activity. Inasmuch as the *anx7*(-/-) mouse mutant is embryonically lethal, we are prepared for the possibility that this mutation may prove to be cytotoxic.

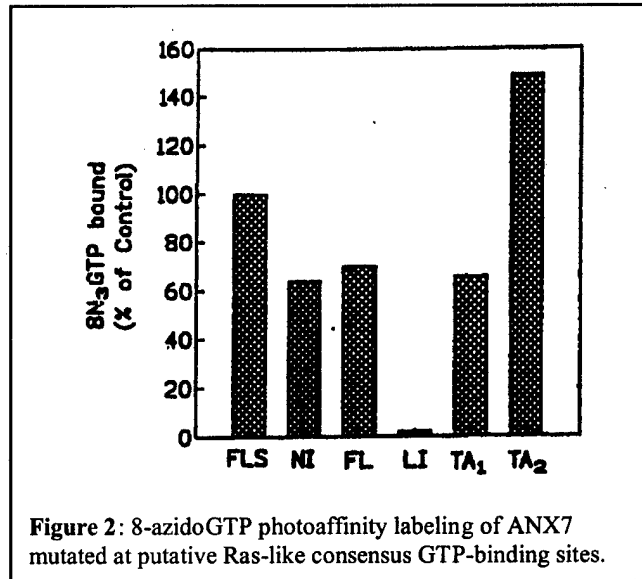
1.a.2. Generation of GTP-binding site mutations in human ANX7

Rationale: ANX7 is a Ca^{2+} -activated GTPase, which contains the five putative RAS-type canonical GTP binding sites. We reasoned that mutating these sites might allow us to test whether GTP might direct any activities of ANX7, including tumor suppression activity.

Approach: Since we did not know which mutations in these GTPase domains might be important for ANX7 activity, we created and expressed mutant ANX7's containing

discrete site-directed RAS-like mutations. These sites in ANX7 were G-2 (QinT); G-4 (NRsN); and G-5 (EiSG). Binding of 8-azido-GTP could then be used to assess GTP binding.

Experiment: Mutations were generated in the putative G-2 Effector or GAP domain either as T148*A (termed TA1, mutation in the cassette exon#6), or as a double mutation, [T148*A, T148A] (termed TA2, with an additional mutation in Exon #7). Other mutations included: a G-4 mutation, N192I (termed NI in Figure 2); another G-4 mutation, N195D (termed ND in Figure 2); a G-5 mutation, L221I (termed LI in Figure 2). One final mutation, F233L (termed FL in Figure 2), a conserved site in all *ras* superfamily members, was also prepared. In *ras* this mutation disorders the GTP binding pocket.



As shown in Figure 2, Western blots showed that substantial amounts of mutant proteins could be prepared. The PhosphorImager data, as well as the graphical representation below, reveal that the LI mutation entirely blocks GTP binding, while NI, TA1 and FL mutants are approximately 60% active. By contrast, the TA2 mutation is approximately 50% activated. "FLS" represents recombinant full-length synexin, or ANX7. Recall that the TA1 and TA2 mutations are in a higher molecular weight ANX7 isoform containing the cassette exon #6, and for that reason run slower on the SDS gel. In RAS, the equivalent LI mutation prevents GTP from binding, just as it does in ANX7.

Interpretation: These data serve to validate the structural basis of the intrinsic GTPase activity of ANX7, and provide mutant anx7 sequences that can be used to assess the importance of the GTPase or GTP binding for tumor suppressor gene activity.

Methods: The wildtype and mutant ANX7 proteins were expressed in the pTrc99A expression system in *E.coli*, and purified to ca. 90% by differential ammonium sulfate precipitation and column chromatography on Ultragel AcA54 (see Cauhuy et al, 1996 for more details). Specific ANX7 content of the 47KDa or the 51KDa bands were estimated by using the ¹²⁵I anti-mouse IgG secondary antibody to label transblotted samples on nitrocellulose that had been bound by primary monoclonal antibody 10E7. ANX7 and ANX7 mutants were photolabeled by 8-N₃-³²[P]-GTP in the presence of 2 mM glutathione to block non-specific binding presently available in our laboratory.

1.a.3. Generation of PKC-binding site mutations in human anx7

Rationale: ANX7 is a Ca^{2+} -activated GTPase, which is activated by PKC and contains two PKC binding sites. We reasoned that mutating these sites might allow us to test whether PKC might direct any activities of ANX7, including tumor suppression activity.

Experiment: Since we did not know which mutations in these PKC binding domains might be important for ANX7 activity, we created and expressed single mutants of ANX7 in each PKC binding site and created double mutants at those sites. We measured the phosphorylation of ANX7 by PKC and found that the double mutants worked the best.

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To test the hypothesis that changes in ANX7 expression levels affects apoptotic processes in cancer cells, we measured cytochrome c release in DU145 cells transfected with wt-ANX7 and the dominant-negative J mutant against the calcium binding site.

2.a.1. Mechanism of ANX7 induced calcium and cytochrome c release

Calcium elevation is a necessary preliminary event in the initiation of the apoptotic cascade (Furuya et al, 1994; Kass and Orrenius, 1999; Scoltock et al, 2000), and compounds that recruit intracellular calcium from the endoplasmic reticulum have therefore been increasingly studied as prototype drugs for induction of apoptosis in cancer cells. An example of such a compound is thapsigargin, which arrests cancer cells in G1/G0 of the cell cycle and induces the apoptotic cascade (Furuya et al, 1994; Lin et al, 1997; Tombal et al, 2000). Thapsigargin raises cytosolic Ca^{2+} concentration by blocking the SERCA-pump ATPase on the ER, thereby blocking reuptake of calcium into the ER. As a further consequence of elevation of cytosolic free calcium, either by thapsigargin or endogenous mechanisms, plasma membrane-localized store operated calcium (SOC) channels are also activated. The latter process contributes to elevation of intracellular calcium, and thus further pushes the cell into the apoptotic cascade.

Large molecules such as proteins have been shown to have similar effects to those induced by thapsigargin. For example, exogenously added $\text{TGF}\beta$ also arrests cancer cells in G1/G0, and induces apoptosis (Furuya et al, 1996). Transfection of the ANX7 gene into the cancer cells also has a very similar sequence of actions (Srivastava and Pollard, 2004). This fact, plus the high prevalence of tumors in the Anx7(+/-) knockout mouse and disorders of calcium metabolism in Anx7(+/-) mouse tissues appear to implicate a specifically thapsigargin-like mechanism for how the ANX7 gene activates human tumor cell apoptosis. For example, in cancer cells, thapsigargin raises cytosolic calcium concentration by preventing reentry of the Ca^{2+} into the ER and activates SOC channels. Similar studies of Ca^{2+} metabolism in beta cells from Anx7(+/-) knockout mice, showed that thapsigargin failed to raise cytosolic Ca^{2+} , and failed to activate SOC channels. The ligand IP3 also failed to release intracellular Ca^{2+} from the ER. So, the reason for the lack of efficacy of thapsigargin in the ANX7(+/-) knockout mouse is a documented ten-fold deficiency in IP3 Receptors. (Srivastava et al, 1999). The importance of IP3 receptors in cancer cells is that IP3 Receptor activation by IP3 is the physiological stimulus needed to release calcium from the ER, thus triggering the mitochondrial permeability transition,

which leads to apoptosis (Szalai, et al, 1999). We have therefore hypothesized that the action of the transfected ANX7 gene on tumor cells, may be to elevate cytosolic Ca^{2+} , and to potentiate subsequent pro-apoptotic actions of the released calcium.

Experiment: Recent studies demonstrate that calcium released from the endoplasmic reticulum synchronizes the mass exodus of cytochrome *c* from the mitochondria, a phenomenon that coordinates apoptosis. Therefore, we examined the effects of altered exogenous *ANX7* expression on metastatic prostate cancer cell line Du145. We found that the effect of the *ANX7* gene on the Du145 cancer cell line enhanced the release of cytochrome *c* by 3 fold into the cytosol in comparison to vector alone control. p53 used as a positive control increased the release of cytochrome *c* by 5 fold into the cytosol (Figure 3).

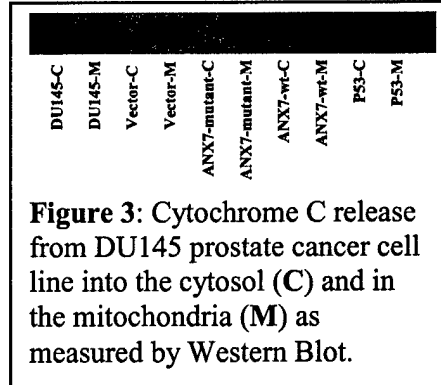


Figure 3: Cytochrome C release from DU145 prostate cancer cell line into the cytosol (C) and in the mitochondria (M) as measured by Western Blot.

Interpretation: The *ANX7* induced apoptotic pathway involves cytochrome *c* release indicating the probable involvement of mitochondria

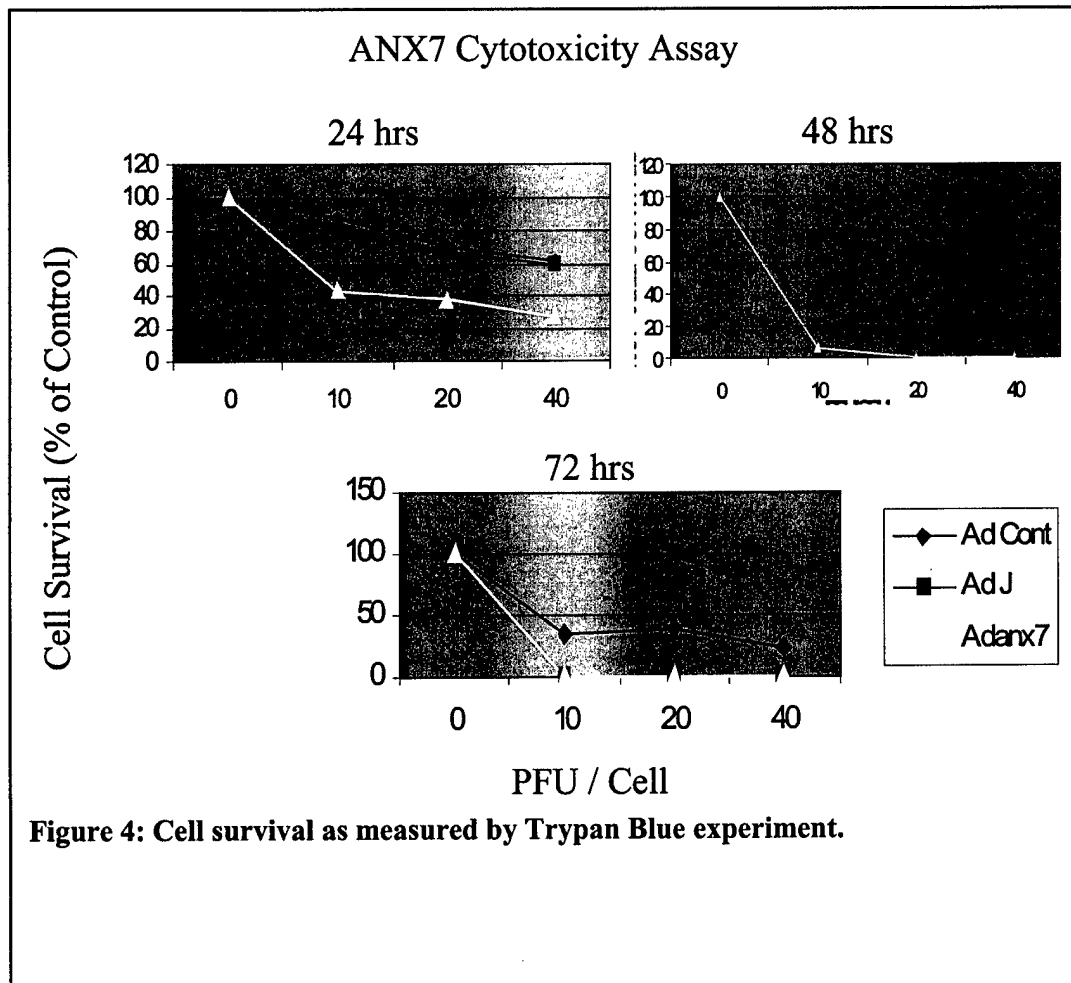
2.a.2. Generation of adenoviral vectors containing wild type *ANX7* and mutants of *ANX7* in calcium, PKC and GTP binding sites and its action on cell proliferation.

Rationale: The underlying hypothesis upon which this aim is based is that the tumor suppression property can be traced to specific “hot spot” amino acid residues in the *ANX7* protein. We also hypothesize that those residues that control tumor cell growth *in vitro* will also control tumor cell proliferation *in vivo*. To test this hypothesis we propose to begin by testing the effect of identified mutations in sites which known to affect *anx7* functions such as Ca^{2+} binding, PKC binding, GTP binding and hydrolysis. For both *in vitro* and *in vivo* cases we also propose to test the effect of constructs which lead to complete loss of function, including antisense *anx7* and a dominant negative *anx7* mutation. If *anx7* is a tumor suppressor gene, then specific residues in the *ANX7* protein ought to exist which are important for the function of suppressing tumor cell proliferation and apoptosis

Approach: We have prepared *ANX7* genes in a recombinant adenovirus expression system which contain tested mutations affecting different *ANX7* functions. These sites include Ca^{2+} binding and GTP binding and hydrolysis. It is quite logical to anticipate that any or all of these functions might be important for suppressing the growth of tumor cells. These mutations are described in section SOW. 1.a. We took the dominant negative mutant *ANX7J* and carried out cytotoxicity assay at different time points and different doses in prostate cancer cells

Experiment: Adenovirus vectors containing either the wildtype *anx7* gene or the dominant negative mutant *ANX7J* were transfected into the Du145 prostate cancer cell line and tested for growth inhibition. Initially, the cells were infected with 10, 20 or 40pfu/cell of either control adenovirus, or adenovirus expressing wildtype or mutant

anx7. The cells were analyzed for their growth at different times after infection. Uninfected cells were analyzed in parallel. Cell growth was monitored and counted using a hemocytometer at 24, 48 and 72 hours. The results which were obtained with trypan blue experiment previously reported had been carried out with adenovirus which was freeze thawed several times and was not giving reproducible results. So, we prepared and purified new batches of adenovectors containing wt-ANX7, ANX7J and p53 at the same time and stored several aliquots in the freezer so there is no discrepancy in the results. The experiments were carried out in triplicate. The cytotoxicity assay revealed that in DU145 cells, at 24 hours vector alone and the dominant negative mutant ANX7J had similar effect, while wt-ANX7 addition had begun killing the cells. However, at 48 hours, introduction of the dominant negative mutant ANX7J began killing the cells at higher pfus and at 72 hours, wt-ANX7 and the dominant negative mutant ANX7J had similar effects (**Figure 4**). These results suggest that the mutation at the calcium binding site has a role to play in killing the cancer cells at the early stages.



On the other hand, in the metastatic B-435 cells, at 24 hours p53 and the dominant negative mutant ANX7J behaved similarly, at 48 hours, the dominant negative mutant ANX7J killed the cancer cells effectively, while p53 did not. These results are in accordance with our finding with human clinical prostate cancer specimens, since high expression of ANX7 was associated with the metastatic disease.

Interpretation: Since the dominant negative mutant ANX7J is against the calcium binding site and the addition of this mutant killed the cancer cells, we conclude that calcium associated function with this identified mutation is mechanistically involved in the tumor suppression phenotype showing metastasis. The effect of the dominant negative mutant ANX7J in killing the cancer cells implies that down-regulation of ANX7 activity could be therapeutic for prostate cancer patients

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3.a. We show that the dominant negative ANX7 down regulated the IP3-Receptor expression in prostate cancer cells

Rationale: The loss of up to 50-60% of endogenous ANX7 protein in the ANX7(+/-) knockout mouse results in a 10-fold loss of IP3 Receptors and complete loss of SOC channels. Since both IP3 Receptors and SOC channels are needed for activating apoptosis, and IP3 Receptor function is the physiological trigger for the mitochondrial permeability transition, it seems reasonable to expect that inactivating ANX7 in prostate cells would down regulate IP3 Receptors.

Experiment: We measured ANX7 and IP3-Receptor mRNA levels in tumor cells treated with adenovirus vector alone, wildtype and dominant negative mutant ANX7. Quantitative RT-PCR was used to quantitate the IP3Receptor

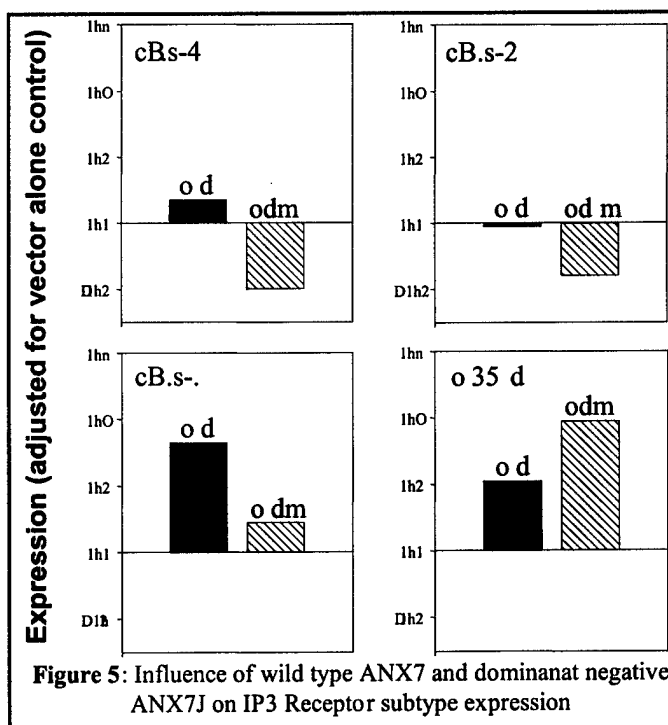


Figure 5: Influence of wild type ANX7 and dominant negative ANX7J on IP3 Receptor subtype expression

messages (types 1, 2 and 3), and levels of beta-actin message was used to normalize levels of RNA used for the analyses. As is shown in **Figure 5**, dominant negative ANX7 down regulated all three IP3-Receptor expression in DU145 prostate cancer cell line.

RT-PCR for IP3 Receptor mRNA subtypes: IP3-Receptors come in three subtypes, in which only a discrete domain varies among the three. The following HUMAN primers were prepared and tested in these experiments.

Type1= [FP:5'-CACCGCGGCAGAGATTGACAC-3'; RP: 5'-CCAGCTGCCCCGAGATTTC-3']

Type2= [FP: 5'-CTGGGGCCAACGCTAATACT-3'; RP: 5'-GAACCCCGTGATTACCTGTGACTG-3'];

Type3= [FP: 5'-GCGGGCCTGTGACACTCTACT-3'; RP: 5'-CGCCGCTCACCAGGGACAT-3'].

We found that Wt-ANX7 expression increased Ad-vector alone control from 0.49 to 0.70 and we observed corresponding increase in IP3R expression. While the dominant negative mutant ANX7J expression reached to 0.80, since it inhibited the activity of endogenous ANX7, we see a decrease in IP3R expression (**Figure 5**).

Interpretation: These results suggests that ANX7 controls all three subtypes of IP3 Receptor expression in DU145 prostate cancer cell line

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4.a.1. To identify the downstream targets of ANX7 that constitute the ANX7 signaling pathway by which ANX7 causes apoptosis and suppresses prostate cancer cell growth.

Rationale: To investigate the genomic mechanisms of wt-Anx7 action in prostate cancer cells, we examined gene expression using cDNA microarrays obtained from Clontech. Our hypothesis is that we can assess the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of prostate cancer cell growth.

Table 1: List of genes that are most affected by ANX7 and p53 and least affected by ANX7J

p	loc	ID
0.013	E04e	interferon gamma antagonist (IFN-gamma antagonist)
0.015	D03a	Ikaros/LyF-1 homolog hlk-1
0.037	C13l	muscarinic acetylcholine receptor M4 (CHRM4)
0.077	C13j	transcription factor Sp1 (TSFP1)
0.085	C04h	HHR23A; UV excision repair protein protein RAD23A
0.099	A02d	A-raf proto-oncogene serine/threonine-protein kinase; PKS2
0.114	F01a	collagen 11 alpha 1 subunit precursor (COL11A1)
0.114	D01h	desmocollin 2A/2B precursor (DSC2); desmosomal glycoprotein II/III
0.122	F03b	fibronectin precursor (FN)
0.126	C02i	G/T mismatch-specific thymine DNA glycosylase (TDG)
0.129	C04k	nuclear factor kappa-B DNA binding subunit (NF-kappaB; NFKB)
0.132	A06m	cell cycle progression 2 protein (CPR2)
0.175	F01c	uroporphyrinogen III synthase
0.186	B14i	vav oncogene
0.206	F03c	arachidonate 5-lipoxygenase

t-test for the difference between A7J and Anx7 in the 3 cancer cell lines, considering only genes that are significantly ($p < 0.15$) affected in cancer and not too much affected by the vector ($\pm 1SD$)

Experiment: In order to assess the downstream targets of ANX7 in prostate cancer cells, we isolated mRNA's from parental prostate cancer cells, prostate cancer cell lines transfected with either vector alone, wild-type, or dominant negative mutant ANX7J. In order to determine the cell type specificity and androgen sensitivity, we chose androgen sensitive metastatic LNCaP, androgen insensitive metastatic PC3 and compared with PREC, a normal prostate epithelial cells from Clonitech. We used p53 as a positive control. We used ATLASTM cDNA expression cancer array to obtain the expression profiles. Comparison of the transcripts between vector control, ANX7J, ANX7 wild type and p53 transfected tumor cells were carried out normal PREC prostate cells. **Table 1** shows the results of the microarray data analysis described in Methods section below. The analysis revealed the genes that are different between A7J and Anx7 in the 3 cancer cell lines, considering only genes that are significantly ($p < 0.15$) affected in cancer and not affected by the vector. On top of the lists are genes related to interferon gamma antagonist, Ikaros/LyF-1 homolog hlk-1, muscarinic acetylcholine receptor M4. **Figure 6** shows the cluster analysis of all the three cancer cells lines and normal cell line at four different conditions. We have used gene expression ratios for normal and cancer cells under conditions of vector alone, Wt-ANX7, ANX7J or p53 and allowed the clustering

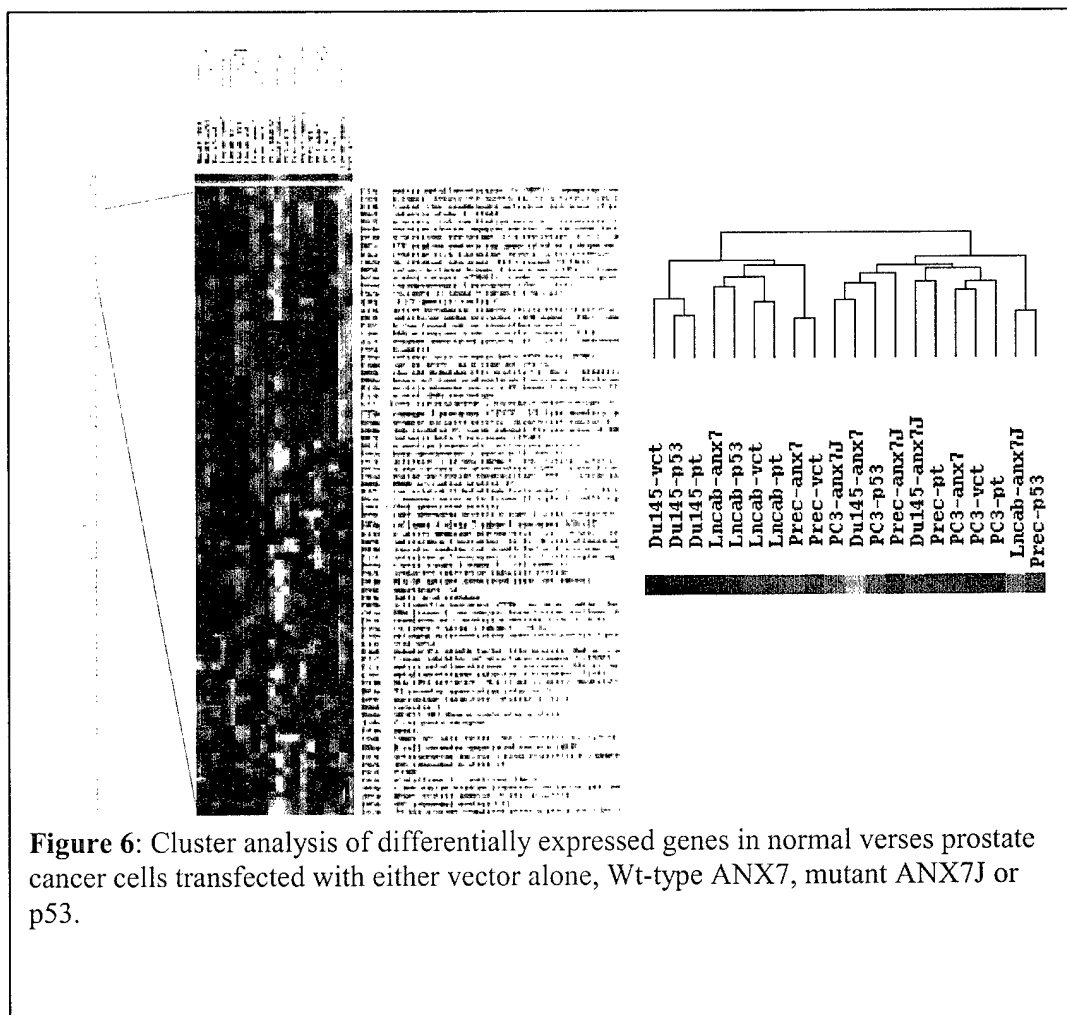


Figure 7: The inset shows the genes that are apoptosis related.

biological/biochemical functions linked to cell proliferation, apoptosis and tumorigenesis.

Methods:

Preparation and labeling of RNA

Total RNAs from prostate normal and cancer cells, either parental or transfected with either vector alone or ANX7, ANX7J or p53 as a positive control were prepared by the method of Champenski et al. and were subjected to DNase 1 digestion to eliminate genomic DNA contamination. Two rounds of purification of poly A+ RNA from total RNA were performed using the mRNA isolation kit from Invitrogen as recommended by the manufacturer. The quality of the RNA were tested by running a formaldehyde denatured agarose gel and quantitated by measuring the optical density at 260nm. A ^{32}P labeled cDNA probe was synthesized from 1 μg of poly A+ RNA from control and tumor samples using MMLV reverse transcriptase, dNTP mix and CDS primer mix comprising the oligonucleotide sequences for the 1200 cancer related genes spotted on the atlas cDNA microarray. The reaction was carried out in a thermocycler set at 50°C for 25 min. and terminated by the addition of 0.1M EDTA, pH 8.0 and 1mg/ml glycogen. The ^{32}P labeled cDNA probe was then purified from unincorporated ^{32}P labeled nucleotides by using a CHROMA SPIN-200 column (clontech) as recommended by the manufacturer. The human atlas cDNA expression array containing 1200 cancer related genes on a nylon membrane was prehybridized using Express Hyb (clontech) at 68°C for 1 hr and hybridized overnight at 68°C with the denatured and neutralized ^{32}P labeled cDNA probe. The membrane was washed three times with 2 X SSC, 1% SDS at 68°C for 30 min. each and twice with 0.1% SSC, 0.5% SDS at 68°C for 30 min. each. The atlas array will be exposed overnight and the results will be compared with the known distribution of genes.

Imaging and quantitation of the cDNA microarray: Imaging data from the Storm PhosphorImager were downloaded into a Microsoft Excel spreadsheet. Duplicate data points were ratio'ed to the ubiquitin standard. Data were then analyzed using the Stanford University ScanAlyze software. These data were also evaluated in parallel with the PSCAN program for point identification and with the JMP program for graphical organization.

Statistical Data mining from cDNA arrays: The first strategy we employed is embodied in the GRASP methodology (Gene Ratio Analysis Paradigm, Srivastava et al, 1999). The GRASP algorithm allows us to specify the changes in specific intensities of given genes which are greater or less than one standard deviation (S.D.) from the average changes of all genes in the entire array. This technique vastly increases the statistical power of the analysis.

We are in the process of validating the results using prostate cancer cells transfected with vector alone, wt-ANX7, ANX7J or p53 at different time points.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of multi-ANX7 expression vectors for evaluation of biochemical functions of ANX7.
- Identification of the dominant negative mutant against calcium and GTP in the anx7 coding domain which are necessary for biochemical functions of ANX7.
- Generation of recombinant adenoviral vectors containing the dominant negative mutants
- Evaluation of altered ANX7 expression in prostate cancer cell growth show that calcium associated function with this identified mutation is mechanistically involved in the tumor suppression phenotype and over expression of ANX7 could prove to be therapeutic
- Evaluation of altered ANX7 expression in prostate cancer cell growth shows further that ANX7 controls all three subtypes of IP3 Receptor expression
- Identification of the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of prostate cancer cell growth using cDNA microarray

REPORTABLE OUTCOME

We showed that regulating ANX7 levels could be therapeutic. In addition, we identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of prostate cancer cell growth using cDNA microarray.

- A manuscript is under preparation.
- The results were presented in USUHS research day, 2003 as part of the plenary session talk and in the poster
- The results were also presented in the "3rd Annexin conference held in Canada, 2003 in the plenary session
- The results formed the preliminary data for the grant, "ANX7 as a molecular target for prostate cancer" that I submitted to NIH on October 1st

CONCLUSIONS

We have generated and identified the dominant negative mutants against calcium and GTP in the *anx7* coding domain which are necessary for biochemical functions of ANX7. We have generated the effective adenoviral constructs containing the dominant negative mutants and wt-ANX7 and have shown that altered ANX7 expression during prostate cancer cell growth involves calcium and over expression of ANX7 could prove to be therapeutic. Additionally, we confirmed the role of calcium by determining ANX7's control on all three subtypes of IP3 Receptor expression. Using cDNA microarray, we have identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of prostate cancer cell growth. Taken together, these data indicate that ANX7 suppresses prostate cancer cell growth and calcium plays a role via IP3-Receptor. In addition, we have identified the members of the ANX7 signaling pathway. Comprehensive effort is underway to validate the results that will further elucidate the ANX7 dependent calcium signaling pathway in prostate cancer cells.

REFERENCES

- Caohuy, H., Srivastava, M., and Pollard, H.B. (1996) GTP-activation of membrane fusion protein anx7 (Annexin VII) and detection of Ca^{2+} -activated GTPase activity in vitro and in vivo. Proc.Nat.Acad.Sci.(USA) 93:10797-10802.
- Creutz, C.E., Pazoles, C.J., and Pollard, H.B. (1978) Identification and Purification of an Adrenal Medullary Protein (Synexin) That Causes Calcium Dependent Aggregation of Isolated Chromaffin Granules. J. Biol. Chem., 253:2858-2866.
- Creutz, E.C., Pazoles, C.J., and Pollard, H.B.(1979) Self-Association of Synexin in the Presence of Calcium: Correlation with Synexin-Induced Membrane Fusion and Examination of the Structure of Synexin Aggregates. J. Biol. Chem., 254:553-558.
- Furuya Y, Lundmo P, Short AD, Gill DL, Isaacs JT The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen independent prostatic cancer cells induced by thapsigargin. Cancer Res (US), Dec 1 1994, 54(23) p6167-75.
- Furuya Y, Ohta S, Shimazaki J. Induction of apoptosis in an androgen-independent mouse cell line by transforming growth factor-beta 1. J Exp Ther Oncol. 1996 Nov;1(6):377-84.
- Kass GE , Orrenius S Calcium signaling and cytotoxicity. Environ Health perspect (US), Feb 1999, 107 Suppl 1 p25-35.
- Lin XS, Denmeade SR, Cisek L, Isaacs JT. Mechanism and role of growth arrest in programmed (apoptotic) death of prostatic cancer cells induced by thapsigargin. Prostate (US), Nov 1 1997, 33 (3) p201-7.
- Raynal, P. and Pollard, H.B. (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium-and phospholipid-binding proteins. BBA Biomembranes 1197: 63-93.
- Scoltock AB, Bortner CD, St J Bird G, Putney JW Jr, Cidlowski JA. A selective requirement for elevated calcium in DNA degradation, but not early events in anti-Fas-induced apoptosis. J Biol Chem. 2000 Sep 29;275(39):30586-96.
- Srivastava, M. Eidelman, O. and Pollard, H.B. (2000). Gene expression microarray pharmacogenomics of wt and mutant CFTR, and of the CF drug CPX. Mol. Medicine 5, 753-767.
- Srivastava, M., Bubendorf, L., L., Nolan, L., Glasman, M., Leighton, X., Koivisto, P., Willi, N., Gasser, T., Kononen, J., Sauter, G., Kallioniemi, O.P., Srivastava, S. and Pollard, H.B. (2001) ANX7, a candidate tumor-suppressor gene for prostate cancer. Proc. Natl. Acad. Sci. 98, 4575-4580

- Srivastava, M., Bubendorf, L., L., Nolan, L., Glasman, M., Leighton, X., Miller, G., Fehrle., W., Raffeld, M., Eidelman, O., Kallioniemi, O.P., Srivastava, S. and Pollard, H.B. (2001) ANX7 as a biomarker in prostate and breast cancer progression. Disease Markers 17, 115-20
- Srivastava, M., Kumar, P., Leighton, X., Glassman, M., Goping, Eidelman, O. and Pollard, H.B. Influence of the Anx7 (+/-) Knockout Mutation and Fasting Stress on the Genomics of the Mouse Adrenal gland. Ann.N.Y.Acad. Sci., 2002; 971:53-60
- Srivastava, M., Eidelman, O. Leighton, X., Glassman, M., Goping, and Pollard, H.B. Anx7 is Required for Nutritional Control of Gene Expression in Mouse Pancreatic Islets of Langerhans. Mol. Medicine. 2002; 8, 781-797.
- Srivastava, M., Glasman, M., Leighton, X, Miller, G., Montagna, C., Reid, T., and Pollard, H.B. Genomic instability and gene expression profile in cancer prone Anx7(+/-) knockout mouse and cell line models. Proc Natl Acad Sci U S A. 2004; 100 , 14287-14292
- Srivastava, M., Bubendorf , L., Raffeld, M., Bucher, C., Torhorst, J., Sauter, G., Olsen, C., Kallioniemi, O.P., Eidelman, E. and Pollard, H.B. (2004) Prognostic impact of ANX7-GTPase in metastatic and HER2 negative breast cancer patients. Clin. Can. Res (In Press)
- Srivastava, M and Pollard, H.B. ANX7-GTPase as Tumor Suppressor: Mechanisms and Therapeutic Opportunities. Trends in Mol. Med (In Press)
- Szalai, G., Krishnamurthy, R., and Hajnoczky, G. (1999) Apoptosis driven by IP(3)-linked mitochondrial calcium signals. EMBO J. 18:6349-6361
- Tombal, B., Weeraratna, A.T., Denmeade, S.R., and Isaacs, J.T. (2000) Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. Prostate 43:303-317.